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STUDIES ON MELANIN—IV

THE ORIGIN OF THE PIGMENT AND THE COLOR PATTERN IN THE ELYTRA OF THE COLORADO POTATO BEETLE (*Leptinotarsa decemlineata* SAY¹)

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INTRODUCTION

AMONG the more important problems in the study of animal pigmentation is the question as to the origin of the color pattern. Perhaps one of the most common of the insects which has a definite color pattern is the Colorado potato beetle (*Leptinotarsa decemlineata* Say), and I have, therefore, investigated the origin of the color in the elytra of this beetle, and have found a possible explanation for the cause of the color pattern.

I have already shown that in all probability the formation of animal pigments is due to the interaction of some chromogen and an oxidase of the tyrosinase type (Gortner, 1910, 1911). I have found evidence which leads me to believe that the same reaction produces the pigment in the potato beetle, and that the color pattern of the elytra is produced by the localized secretion of chromogen.

HISTORICAL

In so far as I am aware the only work which has been done on the nature of colors in *Leptinotarsa* has been reported by Tower. In an article on "Colors and Color Patterns in Coleoptera" (1903), Tower discusses the origin of the pigment in the Colorado potato beetle, and repeats his findings in a larger work, "An Investigation of Evolution in Chrysomelid Beetles of the Genus *Leptinotarsa*" (1906). The biological features of the latter

¹ From the Biochemical Laboratory of the Station for Experimental Evolution. The Carnegie Institution of Washington.

work have been reviewed elsewhere (Cockerell, 1907), but, in so far as I can find, the chemical data and theorizings have been passed over. It is evident from a very casual glance at the chemistry, that Tower has strayed from familiar paths and is wandering far afield. I can refer to only a few of his errors here, but these are so obvious as to show that no reliance can be placed upon the chemical findings, much less upon his chemical theorizings.

Perhaps the worst error, inasmuch as Tower bases all of his results as to the nature of the pigment upon this point, is to state (1906, p. 136), "Bottler, working upon the hair of animals and upon silk, has shown these pigments to be azo compounds, and in insects they belong to the same series." The reference which Tower quotes is a book by Bottler (1902) entitled "*Die animalischen Faserstoffe. Ein Hilfs- und Handbuch für die Praxis, umfassend Vorkommen, Gewinnung, Eigenschaften und technische Verwendung sowie Bleichen und Färben thierischer Faserstoffe. Nach dem gegenwärtigen Standpunkte der wissenschaft bearbeitet.*" From the title I did not expect to find much original work on the subject of *animal pigments*, neither could I find in any of the biochemical literature any reference which could be so construed as to intimate that animal fibers, especially silk, occurred naturally dyed with azo dyes. I find in Bottler's book references to azo compounds in the chapter on "Das Färben animalischer Faserstoffe" and in each instance the references are to azo dyes with which to dye the animal fibers. In almost every instance Bottler gives the origin of the dye employed, as "M. L. Br." (Meister, Lucius and Brüning) or "Fr. Bay." (Fr. Bayer & Co., in Elberfeld) together with explicit directions as to the composition of the dyeing bath. Bottler does mention the naturally occurring pigments by stating on page 36 that "dark-colored wools are usually used in their natural color or dyed dark, as it is only with great

difficulty that they can be bleached": and on page 44 "one uses camel hair in the natural color or dyed dark inasmuch as it can not be bleached." This is the only connection in which the natural-occurring pigments are mentioned.

Tower states (1903, p. 63) that:

The chemical nature of colors is a problem most difficult of investigation, chiefly because such energetic measures are necessary to get the color into solution that there is every reason to suspect that it is no longer the same as that in the cuticula. With solutions of *P. cornutus* in HCl or H₂SO₄, various tests were made, which established the fact that it is much like some of the benzine derivatives, and the spectra of this solution and of permanent brown are identical and closely similar to that of Bismarck brown. There is, however, a great difference between the solution of cuticula color and Bismarck brown, the former being a *colored substance* and the latter a *coloring substance*.

Cuticula colors in acid solution are decolorized by reducing agents such as tin and HCl or strong alkalis. If, however, the decolorized solution is treated with a mild oxidizing agent, part or perhaps all of the color is restored. In the process of decolorization the solution passes from deep brown to lighter shades, to yellow, and eventually to a colorless solution. According to Stecher and others, fast brown is a diazo compound belonging to the group of amidoazo and oxyazo compounds, which form colored solids varying from yellow to deep brown. These diazo, oxyazo, and amidoazo compounds are soluble in alcohol, as is this cuticula color;² and when in solution are rendered colorless by reducing agents, thus forming colorless azo, or hydrazo compounds, which, by mild oxidation, may be reconverted into yellow- or brown-colored diazo, oxyazo, or amidoazo compounds. There is thus a very close agreement between the reaction of cuticula color and the diazo, oxyazo, and amidoazo compounds to reducing and oxidizing agents. In these and in other characters the cuticula colors resemble these benzine derivatives, but not in any respect do they resemble other colored substances or dyes known to organic chemistry. The existence of azo compounds has been recognized by Bottler in the hairs of animals and in silk fibers, where they function as pigments and have some of the structural peculiarities of cuticula color. I feel perfectly certain, therefore, that these cuticula colors are azo compounds, a conclusion based upon the following characters: (1) their colors; (2) solubility;

² On page 41 of the same work Tower states that these cuticula colors are "insoluble in water, alcohol, ether, oils, weak acids or alkalis. Soluble in strong concentrated mineral acids with dissolution of the cuticula."

(3) behavior to oxidizing and reducing agents; (4) crystallization; (5) spectrum; and (6) their decomposition products.

Let us see what of real value these characters possess:

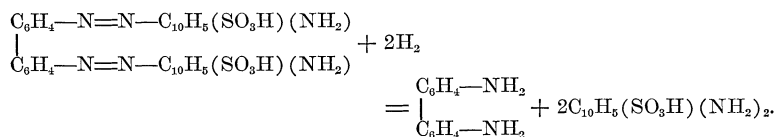
1. If color is any criterion, all colored objects must contain azo dyes, inasmuch as azo compounds occur in almost every possible shade of color,

2. If the data which Tower gives on page 11 (1903) be referred to we find

Cuticula colors	$\left\{ \begin{array}{l} \text{Black} \\ \text{Dark brown} \\ \text{Brown} \\ \text{Straw yellow} \end{array} \right\}$	Located in primary cuticula	$\left\{ \begin{array}{l} \text{Permanent. Insoluble in water, al-} \\ \text{cohol, ether, oils, weak acids or} \\ \text{alkalis. Soluble in strong concen-} \\ \text{trated mineral acids with dissolu-} \\ \text{tion of the cuticula.} \end{array} \right\}$
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These are not the characteristics of the azo compounds, for azo compounds are soluble in alcohol and in many cases are very soluble in water. The "amidoazo" compounds are soluble in weak acids and the oxyazo compounds are very soluble in weak alkalis. *The characters which Tower gives are, however, exactly those characters which are possessed by the melanins.*

3. Tower has made a great mistake when he states that azo compounds are reduced to colorless bodies and then reoxidized to colored diazo, oxyazo or amidoazo compounds by mild oxidizing agents. *When azo compounds are reduced by tin and hydrochloric acid the azo union is broken, each nitrogen atom yielding an amino group, and the colored compound can not be regenerated by mild oxidation, but the original diazotization and coupling must be repeated before a color results.* For example we reduce Congo red and we get benzidine and "naphthionic acid" neither of which gives a color on mild oxidation. (See Mulliken (1910), p. 26, footnote.)



There are, to be sure, dye stuffs (for example, indigo), which do reduce to colorless compounds, or leuco bases,

and which reoxidize to the original color and compound, *but the azo compounds are distinguished from these colors by the fact that no reoxidation takes place.*

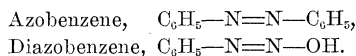
4. I have been unable to find any data as to the crystal form of cuticula pigments. All writers report that they are non-crystalline. If Tower succeeded in preparing crystals he has succeeded where every one else has failed. The azo compounds, on the other hand, are usually easily obtained in crystal form.

5. As to the value of the spectrum I will quote Tower's own words (1903, p. 56).

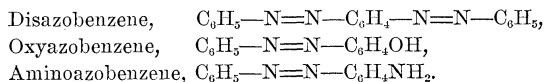
The study of animal and plant pigments has been carried on almost entirely by means of spectrum analysis. . . . There is, however, very grave doubt as to the value of such spectrum work. After spending several months in the analysis of cuticula pigment of Coleoptera, Lepidopterous larvæ, and Hymenoptera, I discarded as worthless all of my results, because in no case was I able to obtain a spectrum of sufficient permanence, or one that had absorption bands of enough constancy, to be reliable. The same solution with the same apparatus and light will vary in its spectrum from day to day. Moreover, any slight change in acidity or alkalinity, or in dilution or concentration, etc., will produce a variation in the spectrum.

6. In regard to the decomposition products of cuticula pigments, I can find no data in Tower's paper, so that I do not know in what respect they may resemble known organic compounds. Enough has been shown, however, to prove that Tower's statement (1906, p. 136), "we are safe, then, in concluding that these cuticula pigments are azo compounds, but whether they are azo, diazo, oxyazo, or amidoazo³ is not known," is entirely without foundation.

³ For those who are not familiar with this nomenclature I will give here an example of the formula of each class:



Tower probably refers to *Disazo* compounds, inasmuch as diazo compounds are very unstable and are not coloring matters.



Other examples of this chemistry are numerous, but I can only mention one or two. Tower states (1903, p. 54) "Sundwick and Ledderhose conclude that chitin is an amido derivative of *a carbohydrate having the formula* $C_6H_{100}O_{50}$." ⁴ I was willing to allow this as an error in proof-reading had not the same $C_6H_{100}O_{50}$ occurred again on page 55, where he adds "*and hence glucoside, like cellulose,* ⁵ and other plant substances."

Again I quote from the 1906 paper (p. 123) "Griffiths farther isolates and studies a green pigment which is allied to uric acid or is a uric acid derivative called lepidopteretic acid ($C_{11}H_{10}Az_2N_8O_{10}$?)." I have not seen Griffiths's paper (1892), but, from the formula which Tower gives, I would say that the question mark is well placed, inasmuch as the English form of nitrogen is not known to differ from the French variety. Some of the other errors will be referred to in connection with my work.

EXPERIMENTAL

The Formation of the Pigment by Oxidation.—The material employed was the pupæ and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* Say. Large larvæ were secured in the field and placed in battery jars, provided with abundance of fresh potato stalks, and containing about four inches of sifted soil in the bottom. The top of the jar was covered with mosquito netting. Within a few days all of the larvæ had entered the soil and had pupated. When the adult beetles emerge from the pupal skin, the elytra are an almost uniform light yellow color, and upon this surface the lines of the darker color pattern shortly appear. In accordance with my former work I have found that this pigmentation is an oxidation, induced by an oxidase of the tyrosinase type.

Tower (1903, p. 58) states that he tested the rate of pigmentation when the pupæ were kept in an atmosphere

⁴ My italics.

⁵ My italics. Cellulose is in no way related to the glucosides.

containing respectively "O₂ 40 parts, N₂ 80 parts; O₂ 60 parts, N₂ 80 parts; O₂ 80 parts, N₂ 80 parts, and O₂ pure. In the first two no changes were noted, but in the third there was a large mortality, showing that the amount of O₂ had become toxic and the pigmented areas were small and weak. The same results were attained in a more marked manner with pure O₂."

Tower's results in this instance are easily accounted for, inasmuch as a very small quantity of oxygen is consumed in the process of pigmentation and even in normal air the oxygen is present in enormous excess. The rate of pigmentation is, in all probability, at a maximum even when the oxygen is present in very small concentration. Tower further adds (1903, p. 58): "A second set of experiments consisted in diminishing the O₂ present, but even although O₂ was absent, pigmentation was not changed. A third set consisted in placing pupæ in an atmosphere of CO₂, N, H, and pigmentation, if about to begin or already begun before the pupæ were placed in the gas, was not retarded or changed." Tower does not state what precautions were used to ensure the entire absence of oxygen. *I have found that when all oxygen is absent, no pigmentation takes place.*

Newly emerged adults, whose elytra showed no trace of brown pigment, were placed in gas wash bottles which were provided with a tubulated ground glass stopper, arranged in such a manner that when the stopper is turned slightly the stream of gas is cut off and the bottle is tightly closed (see Eimer and Amend's 1910 Catalogue no. 3658). A current of washed carbon dioxide, from a Kipp apparatus, was then passed through the bottle for twenty-four hours and then the gas was shut off by turning the stopper slightly. *In every instance the elytra of the beetles remained colorless as long as they remained in the carbon dioxide and if removed to the air, or else if the carbon dioxide were displaced by a stream of pure oxygen, before decomposition set in, pigmentation pro-*

ceeded at once. Other beetles were kept in the air as a check upon the results, and in some instances one elytron was removed, and either the beetle minus one elytron was placed in the carbon dioxide and the other elytron kept in the air as a check, or *vice versa*. The same results were obtained when pure hydrogen was used instead of carbon dioxide. I found that it was impossible to obtain entire absence of oxygen when either rubber or cork stoppers were employed, for on long standing some traces of oxygen, apparently, diffuse through the rubber or cork, and cause pigmentation to proceed.

If a beetle with unpigmented elytra be kept submerged in water by means of a small weight, for example in a test tube under a ten cent piece, no coloration appears in thirty-six hours, while in a check the pigmentation was complete in twelve hours. When, however, the beetles were removed from the water and exposed to the air, pigmentation proceeded at once. In most cases the beetles revived after having been submerged for thirty-six hours, as did those that had been forty-eight hours in carbon dioxide. Heating an unpigmented elytron at 70° for one minute totally inhibits pigment formation. The above results are identical in every respect with those which I have reported in the case of the periodical cicada (1911) and the meal worm (1910 b.), so that there can be no doubt that the formation of the pigment in the elytra of the Colorado potato beetle is an oxidation. Tower states (1903, p. 53) that the cockroach, *Phyllodromia germanica* Linn. was used in contributing to his conclusions, and Phisilax (1905) has since shown that in this material the pigmentation is due to an oxidation induced by a tyrosinase.

Evidences of Enzyme Action.—Tower claims to have shown the presence of enzymes by finding *stained zymogen granules* in some of his sectional material. He states (1903, p. 60), “it was impossible to prove the existence of a zymogen in the hypodermis of much of my

material, as almost all of the best of it was in sublimate-acetic-acid fixation, which does not fix zymogens well, but dissolves them and leaves vacuoles where the granules should have been. . . . With Berenyi and picric acid mixtures some zymogen granules were preserved, but the rest of the material was so bad that the preparations were useless. The account of the zymogens and their part in pigmentation is based on material from *L. decemlineata* and *C. femorata*. For demonstrating the presence of substances, probably zymogens, I used iron hæmatoxylin, which is fairly good, and Bensley's stain for zymogens."

I do not know by what right one can designate a stained granule as a zymogen when we do not know whether zymogens exist as distinct bodies or not. Zymogen is only a term which we use to state the fact that in some cases there is, apparently, the sudden appearance of enzyme action where no enzyme action could be previously detected. We call the mother substance of this enzyme a zymogen, but since the term "enzyme" is only another term for a peculiar kind of energy, which may some day be shown to be only a catalysis, or other physical phenomenon, we have, at present, no basis for believing that either enzymes or zymogens can be stained by definite stains.

Tower states that he has prepared an enzyme which is responsible for the production of pigment, and which causes the hardening of the primary cuticula. To this "enzyme" he has given the name "chitase." The method of preparing this enzyme is given on page 60 (1903), and on page 138 (1906). Pupæ were ground with quartz sand and the mixture extracted with 35 per cent. alcohol containing 2 per cent. of acetic acid, or with 50 per cent. alcohol containing 10 per cent. of glycerol. "Dilute alcohol and acetic acid served the best, as it gave the largest precipitate upon the addition of 95 per cent. alcohol. The white, rather flocculent, precipitate pro-

duced was separated by filtration, redissolved in alcohol and acetic acid, and reprecipitated several times, and finally used in 30 per cent. alcohol containing one tenth per cent. acetic acid." Into this solution were suspended pieces of primary cuticula, taken before pigmentation had begun to develop. "The result was that in about thirty-six hours the cuticula had become brown, being first drab, then pale brown, then full brown. Beyond this, coloration did not advance. It was uniform over the entire surface, but, although areas where spots normally develop were present, no tendency to spot formation was observed."

This does not prove that the solution which Tower prepared contained enzymes, for the pieces of unpigmented cuticula which were used as the testing material may have contained sufficient enzymes to produce the pigmentation. Tower does not mention that he used a check of distilled water in place of the "enzyme" solution. I have repeated this part of the work, and for the piece of the primary cuticula I have used the fresh elytron, taken before the pigmentation had begun to develop. I find that there is an appreciable difference in the rate of the development of the pigment when the elytron is placed in the "enzyme" solution or in distilled water, *and that this difference is in favor of the distilled water.* The color is distributed more over the entire surface in the case of the enzyme solution, but I find that this is probably due to the fact that the precipitate contained some of the chromogen and when this came in contact with the tyrosinase on the surface of the elytron, pigment was produced. *Tower's "enzyme" solution, if it does contain an enzyme, does not contain the enzyme which causes the pigmentation, for it gives none of the tests for oxidases.* Oxidases are destroyed by solutions that are slightly acid so that this is probably one of the reasons why his preparations did not give more positive results. Another reason could be found

in the high percentage of alcohol which was used both to extract the enzyme, and as a solvent.

The Isolation of a Tyrosinase.—Phisalix (1905), Dewitz (1902), Roques (1909) and Durham (1904), as well as myself (1910 b.) (1911), have shown that the production of melanin is caused by the action of an oxidizing enzyme of the tyrosinase type upon some oxidizable chromogen, which may, in some instances, be tyrosin. My first step was, therefore, to ascertain whether or not tyrosinase were present in the potato beetle. I found that it was present in large amounts in the beetles that were collected in the field. In order to make sure that there was no mistake, I tested some of the potato leaves, and there I found abundance of tyrosinase. I found, however, that if I removed large larvæ to the laboratory and kept them without food until they pupated, that the body contents of these three or four days old pupæ gave no test for the enzyme. As a final precaution, however, I used only the unpigmented elytra of beetles which had transformed from such pupæ. In this manner there seems to be no possibility that the food could have contaminated the material, for a period of a least twelve days without food must have elapsed between the securing of the larvæ and the removing of the unpigmented elytra from the adult beetles. The elytra, which showed only a faint trace of the color pattern which was to develop, were ground with quartz sand in an agate mortar, and the mixture leached with distilled water containing a few drops of chloroform. The filtered solution was clear, gave an intense blue with a drop of tincture of gum guaiac, produced a rapid darkening in solutions of tyrosin, leading to the deposition of the typical black precipitate, lost its activity at 70°, or in the presence of *meta* di phenols [see Gortner (1911 b.)], and behaved in every way identically like other preparations of tyrosinase which I have reported from other sources. *There can be no doubt that if the pigmentation in other instances is*

caused by the action of a tyrosinase, the same cause produces the color pattern in *Leptinotarsa*.

The Cause of the Color Pattern on the Elytra.—If the pigmentation is due to the oxidation of a chromogen by an enzyme, the color pattern may develop in four ways. (1) The enzyme may be present over the entire body and the chromogen may be localized. (2) The enzyme may be localized and the chromogen secreted over the entire body. (3) Both enzyme and chromogen may be localized in those spots which form the color pattern. (4) Those spots which lack color may be pigmentless because there is an inhibitor to pigment production present. I have found that in all probability the first hypothesis is the true one, for, although the pigmentation is not much more general when an unpigmented elytron is placed in a solution of tyrosinase, when such an elytron is floated upon a solution of tyrosin the entire elytron becomes pigmented. Apparently, therefore, the chromogen is localized and the enzyme is secreted over the entire surface.

SUMMARY

It has been shown that Tower's statements as to the nature of the cuticula pigments, and the methods of pigment formation, are based upon wrong interpretations of his results, and upon errors in the application of chemical data. The cuticula pigment is not an azo compound but belongs to the melanins.

The pigmentation of the elytron of the Colorado potato beetle (*Leptinotarsa decemlineata* Say) is produced by the interaction of an oxidizing enzyme of the tyrosinase type, and an oxidizable chromogen. The color pattern is caused by the localized secretion of chromogen.

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